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Cryptic introgression into the Kidney saxifrage (*Saxifraga hirsuta*) from its more abundant sympatric congener *Saxifraga spathularis*, and the potential risk of genetic assimilation

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Running title: Cryptic introgression in *Saxifraga hirsuta*

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- 1 • *Background and Aims* Although hybridization can play a positive role in plant
2 evolution, it has been shown that excessive unidirectional hybridization can result in
3 replacement of a species' gene pool, and even the extinction of rare species via genetic
4 assimilation. In the present study, we have examined levels of introgression between
5 the common *Saxifraga spathularis* and its rarer congener *S. hirsuta*, which have been
6 observed to hybridize in the wild where they occur sympatrically.
- 7 • *Methods* Seven species-specific single nucleotide polymorphisms (SNPs) were
8 analyzed in 1025 plants representing both species and their hybrid *S. x polita* from 29
9 sites across their ranges in Ireland. In addition, species distribution modelling was
10 carried out to determine whether the relative abundance of the two parental species is
11 likely to change under future climate scenarios.
- 12 • *Key results* *S. spathularis* individuals tended to be genetically pure, exhibiting little or
13 no introgression from *S. hirsuta*, but significant levels of introgression of *S.*
14 *spathularis* alleles into *S. hirsuta* were observed, indicating that populations exhibiting
15 *S. hirsuta* morphology are more like a hybrid swarm, consisting of backcrosses and
16 F₂s. Populations of the hybrid, *S. x polita*, were generally comprised of F₁s or F₂s,
17 with some evidence of backcrossing. Species distribution modelling under projected
18 future climate scenarios indicated an increase in suitable habitat for both parental
19 species.
- 20 • *Main conclusions* Levels of introgression observed in this study in both *S. spathularis*
21 and *S. hirsuta* would appear to be correlated with the relative abundance of the
22 species. Significant introgression of *S. spathularis* alleles were detected in the
23 majority of the *S. hirsuta* populations analysed and, consequently, ongoing

1 introgression would appear to represent a threat to the genetic integrity of *S. hirsuta*,
2 particularly in areas where the species exists sympatrically with its congener and
3 where it is greatly outnumbered.

4

5 **Key words:** genetic assimilation; hybridization; introgression; population genetics;
6 *Saxifraga hirsuta*; *Saxifraga spathularis*; single nucleotide polymorphisms; species
7 distribution modelling

INTRODUCTION

Hybridization is a common phenomenon in plant taxa, and can result in a wide range of potential evolutionary consequences, both positive and negative (Barton 2001). The process can give rise to the evolution of new species (Anderson & Stebbins 1954; Seehausen 2004), and introgression can act as a source of potential adaptive variation (Lewontin & Birch 1966), but excessive unidirectional hybridization can result in replacement of a species' gene pool (Beatty, Philipp & Provan 2010) and has even been implicated in the extinction of rare species via genetic assimilation (Levin, Francisco-Ortega & Jansen 1996). It is thus clear that no single evolutionary trajectory results from the process of hybridization, and the unpredictability of the final outcomes has led to ongoing interest from researchers.

In recent years, global climate change has led to shifts in the distribution ranges of many species (Parmesan & Yohe 2003; Hickling *et al.* 2006; Kelly & Goulден 2008). One consequence of these range shifts is the creation of novel species assemblages. Such ecosystem reorganizations offer new opportunities for hybridization between species that would otherwise remain isolated from each other, both ecologically and reproductively (Garroway *et al.* 2010; Muhlfeld *et al.* 2014; Potts *et al.* 2014). In addition to observational studies on emergent hybridization, species distribution modeling approaches are now being employed to determine the role of future range dynamics in the potential creation of new hybrid zones (Sanchez-Guillen *et al.* 2014). To date, however, these models have not been applied to assess the possible impacts of climate change on existing known hybrid zones in the context of potential genetic assimilation.

1 *Saxifraga spathularis* Brot. and *S. hirsuta* L. are both members of the Lusitanian flora,
2 whose disjunct distribution between Ireland and northern Spain has been of great intrigue
3 to botanists (Webb 1983; Beatty & Provan 2013, 2014). Both species belong to the
4 *Saxifraga* Section *Gymnopera*, which also includes two other members, *S. umbrosa* L.
5 and *S. cuneifolia* L. *S. umbrosa* readily hybridizes with both *S. spathularis*, resulting in
6 *S. x urbium*, an artificially induced/propagated hybrid commonly referred to as London
7 Pride, and with *S. hirsuta*, resulting in *S. x geum* L., a wild naturally occurring hybrid,
8 found where the ranges of the two parental species overlap in western Pyrenees (Webb &
9 Gornall 1989). *S. cuneifolia*, however, does not form hybrids with any of its congener
10 species, either in the wild or artificially. The two Lusitanian species exhibit contrasting
11 patterns of occurrence throughout their divided ranges. In the Iberian reaches of their
12 range, *S. hirsuta* is more commonplace, stretching from the mountains of Galicia and
13 Andalusia in the northwest, across northern Spain to the Pyrenees, compared to the more
14 limited range of *S. spathularis*, which is restricted to the northwestern corner of Iberia. In
15 Ireland, however, levels of abundance are reversed: *S. spathularis* is significantly more
16 abundant and wide-ranging, being found throughout the south and west of the country,
17 primarily Counties Galway, Kerry, Cork and Waterford, whilst *S. hirsuta* is limited to the
18 extreme southwest in Counties Cork and Kerry. Both species have similar habitats
19 although *S. spathularis* reaches a greater altitude and *S. hirsuta* is less tolerant of exposed
20 sites. A putative hybrid between the two species, *S. x polita* (Haw.) Link, has been
21 identified in both the Irish and Iberian parts of their range. Records of *S. x polita* are rare
22 in Iberia compared to Ireland, most likely due to the relatively limited area of overlap in
23 the parental species' ranges in Iberia, and the fact that where they do, they tend to occupy

1 different environmental niches, (McGregor, 2008). In Ireland, however, the hybrids are
2 common where both species co-occur in Counties Cork and Kerry and, interestingly, also
3 in Co. Galway, where only one of its parental species, namely *S. spathularis*, occurs
4 (Webb 1951).

5 Given the previously documented threats of genetic assimilation by a more abundant
6 congener in other plant species, and the relative abundances of *S. hirsuta* and *S.*
7 *spathularis*, along with the occurrence of the hybrid *S. x polita* in Ireland, the questions
8 addressed in the current study were: (1) Is there evidence of introgression of *S.*
9 *spathularis* alleles into *S. hirsuta*? (2) If so, to what level is the introgression occurring
10 and is it placing the gene pool of the rare *S. hirsuta* under threat of genetic assimilation?
11 (3) What is the genetic composition of *S. x polita* hybrid populations? (4) Is the
12 distribution of the two parental species likely to change under future climate scenarios,
13 and thus increase or decrease the likelihood of hybridization?

MATERIALS AND METHODS

Study species

Saxifraga spathularis (St. Patrick's cabbage) and *S. hirsuta*, (Kidney saxifrage) are perennial stoloniferous herbs. The two species and their hybrid (*S. x polita*) can be distinguished by relative leaf shape, pubescence tothing, and the width of the cartilaginous margins of the leaf lamina (Gornall 1998). *S. spathularis* leaves are extremely distinctive, with smooth, thick, waxy, spathulate leaves, each of which has a sharply toothed margin. The leaves of *S. hirsuta* are soft and hairy, and are fringed with a crenate tothing, with petioles that are long thin and hairy. The leaves of *S. x polita* exhibit an intermediate form, both in terms of relative leaf shape and tothing of the leaf margin. The width of the cartilaginous margins of the leaf lamina in *S. spathularis* is 0.1mm, for *S. hirsuta* it ranges between 0.1-0.2mm, while for *S. x polita*, an intermediate range of between 0.1- 0.15mm has been recorded (Gornall, 1998). The leaves of all three congeners form a basal rosette/turf, from which the erect flowering stem projects. All three taxa flower from May through to July, when the stem bears panicles of star-shaped, five-petaled white/pink flowers. The flowers are insect pollinated and seeds are primarily wind dispersed.

Sampling and DNA extraction

Samples were collected from 29 locations across the ranges of *Saxifraga spathularis*, *S. hirsuta* and *S. x polita* in Ireland (Figure 1; Table 1). These included two sites in Co. Galway, where both *S. spathularis* and *S. x polita* were present, but where no verified

records of *S. hirsuta* exist (Stelfox 1947), as well as samples of *S. spathularis* from Co. Waterford, where neither *S. hirsuta* nor *S. x polita* are present. DNA was extracted using the CTAB method of Doyle and Doyle (1987).

Single nucleotide polymorphism (SNP) ascertainment and genotyping

Species-specific single nucleotide polymorphisms (SNPs) were developed from an ascertainment set containing four individuals of each of the two parent species using the ISSR cloning method outlined in Beatty, Philipp & Provan (2010). Allele-specific PCR (AS-PCR) primers were designed as described in Provan *et al.* (2008), and SNP analysis carried out as described in Beatty, Philipp & Provan (2010). In total, 1025 individuals were genotyped for seven SNP loci (Table 2).

Data analysis

As populations studied comprised a mixture of parental species and hybrids, and thus were unlikely to be close to Hardy-Weinberg equilibrium, the commonly used software package STRUCTURE (Pritchard, Stephens & Donnelly 2000) was not used. Instead, the percentage of alleles from each parental species found in each individual was represented as a stacked histogram as suggested by Hauser *et al.* (2012). To further investigate the genetic composition of hybrid individuals, we used the program NEWHYBRIDS (V 1.1b; Anderson & Thompson 2002). As suggested by the authors, the number of generations was restricted to two, which gave six classes of genotypes (both parents, F₁, F₂, BC_S. *spathularis* and BC_S. *hirsuta*). The program was run with the nuclear SNP data using 50,000

burn-in iterations followed by 500,000 Markov Chain Monte Carlo iterations using default priors for allele frequencies and mixing proportions.

Species distribution modelling

Species distribution modelling was carried out to determine suitable future climate envelopes for both species using the maximum entropy approach implemented in the MAXENT software package (version 3.3.3; Phillips & Dudik 2008). Species occurrence data in Ireland between 1950 and 2000 (330 and 181 occurrences for *S. spathularis* and *S. hirsuta*, respectively) were downloaded from the Global Biodiversity Information Facility data portal (<http://www.gbif.org/>). Current-day climatic data (1950–2000; Hijmans *et al.*, 2005) at 2.5-minute resolution were clipped to encompass the island of Ireland (i.e. 10.75° W to 5.20° W, and 51.35° N to 55.55° N) to reduce potential problems associated with extrapolation. Models were generated using cross-validation of 10 replicate runs under the default MAXENT parameters. Model performance was assessed based on the area under the receiver operating characteristic curve (AUC). Models were projected onto climate data for the years 2050 and 2080 generated under the UKMO-HADCM3 model based on three different emissions scenarios (A1b, B2a and A2a; www.ccafs-climate.org). Outputs from the three models were averaged to give a single consensus model for each species at each time period.

RESULTS

Genetic analyses

Analysis of 1025 individuals from all three species, including potential hybrid *S. x polita* populations, indicated a broad spectrum of intra-individual allele frequencies, ranging from 100% *S. spathularis* to 100% *S. hirsuta* , with all levels of intermediate allelic composition and hybrid classification evident (Figure 2). *S. spathularis* individuals tended to be largely genetically pure, exhibiting little or no introgression from *S. hirsuta*. Most individuals were either 100% *S. spathularis*, and less than 5% of individuals exhibited more than two *S. hirsuta* alleles. This was reflected in the NEWHYBRIDS analysis, which assigned the majority of the individuals clearly to *S. spathularis*, and only four as having a majority chance of falling onto the BC_{*S. spathularis*} class. In total, *S. hirsuta* alleles only accounted for 4.7% of the *S. spathularis* gene pool of the populations analysed. There was much greater evidence for introgression of *S. spathularis* alleles into *S. hirsuta* populations, with less than 15% of individuals exhibiting 100% *S. hirsuta* alleles. Overall, the level of introgression of *S. spathularis* alleles into *S. hirsuta* populations was 19.2%, significantly higher than the opposite scenario (Mann-Whitney test; $z = -14.37$, $P < 0.0001$). Six individuals exhibited *S. spathularis* alleles at a frequency greater than 0.5. The NEWHYBRIDS analysis showed high levels of assignment to the BC_{*S. hirsuta*} class, indicating that populations exhibiting *S. hirsuta* morphology are more like a hybrid swarm, also containing some F₂s. Populations of the hybrid, *S. x polita*, were generally comprised of F₁s or F₂s, with some evidence of backcrossing. All

1 hybrid individuals exhibited a mixture of alleles from both parental species. In total, *S.*
2 *spathularis* alleles were slightly predominant, at a frequency of 0.536.

3

4 *Species distribution modelling*

5 The mean AUC values (0.881 [sd = 0.034] and 0.957 [sd = 0.037] for *S. spathularis* and
6 *S. hirsuta* respectively) indicated a prediction that was far better than random. The
7 present-day (2000) models for both species were an accurate representation of their actual
8 distributions, and projected future distributions indicated an increase in suitable habitat
9 for both species (Figure 3). A comparison of the area in southwest Ireland, where both
10 species are currently found sympatrically, indicated an increase in suitable cells of *ca.*
11 18% for *S. spathularis* between 2000 and 2080, and an increase of *ca.* 91% for *S. hirsuta*
12 over the same time period (Figure 4). The proportion of suitable cells for *S. hirsuta* that
13 were also suitable for *S. spathularis* rose from 94% in 2000 to 100% in 2050 and 2080.

DISCUSSION

The present study further highlights the utility of species-specific SNPs in studies into hybridization in plant taxa, indicating the occurrence of cryptic introgression into both *S. spathularis* and *S. hirsuta*, but particularly the latter. Nevertheless, despite developing primer pairs from the ISSR libraries to amplify 96 sequence-tagged sites (STS), we only managed to develop seven sets of AS-PCR primers that could consistently be used to genotype SNPs. This was mainly due to difficulties in amplifying the orthologous locus in *S. hirsuta* using primers developed from the *S. spathularis* ISSR library, even after relaxing PCR conditions by lowering the annealing temperature. This was surprising, given the fact that they are sister species (Sanna 2013), and given that cross-genus amplification of orthologous loci was possible using the same ISSR-based approach in *Pyrola* (Beatty, Philipp & Provan 2010). Furthermore, it should be borne in mind that genetically more similar orthologues might be more likely to undergo recombination, thus facilitating introgression.

The findings of this study would seem to indicate that levels of introgression are primarily density-dependent. This is most apparent in Cleanderry Wood (Site 17, Figure 2), which is the only location that harboured a large proportion (15/22) of genetically pure *S. hirsuta*, and where no populations of either *S. spathularis* or *S. x polita* were found in the vicinity. Similarly, the majority of *S. hirsuta* collected from Site 10 in Glengariff Forest Park, where only two *S. spathularis* plants were found, exhibited 100% *S. hirsuta* alleles. Further evidence of this is seen at sites where hybrids are found with only one of the parental species. Significantly different frequencies of *S. spathularis*

1 alleles were found in hybrid populations from Connemara National Park (Site 27, Figure
2 2), where *S. hirsuta* has never been recorded (0.629), compared to Dromagowlane (Site
3 14, Figure 2), where *S. spathularis* is absent (0.347; Mann-Whitney test; $z = -5.53$, $P <$
4 0.0001). Such density-dependent introgression has previously been cited as a threat to
5 the persistence of a rare species sympatrically with a more abundant congener (Ellstrand
6 & Elam 1993; Burgess *et al.* 2005; Chan *et al.* 2006; Lajbner *et al.* 2009; Beatty, Philipp
7 & Provan 2010).

8 Individuals identified as *S. x polita* all contained a mixture of parental alleles, with *S.*
9 *spathularis* allele frequencies ranging from 0.214 to 0.928. The majority were assigned
10 in the NEWHYBRIDS analysis to F₁ or F₂ classes, with some backcrosses to both *S.*
11 *spathularis* and *S. hirsuta*. This is consistent with the observation that wild hybrids
12 within Section Gymnopera (*S. x polita* in Ireland and *S. x geum* in the western Pyrenees)
13 are fertile, and form hybrid swarms that display a full range of intermediate morphologies
14 between the relevant parental species (Webb 1951; McGregor 2008). Given the apparent
15 fertility of hybrids, and indications of density-dependent introgression, there is thus the
16 real chance that, over time, the rarer *S. hirsuta* could be under threat of genetic
17 assimilation by the more abundant *S. spathularis* (Levin, Francisco-Ortega & Jansen
18 1996; Beatty, Philipp & Provan 2010). Indeed, over 50 years ago, Webb had already
19 highlighted the fact that *S. hirsuta* was rarer than the hybrid *S. x polita*, and that "... *S.*
20 *hirsuta* could not be maintained as a distinct species" (Webb 1951; Page 204).

21 The occurrence of populations of *S. x polita* in Co. Galway, where only one of the
22 parental species – *S. spathularis* – is present, is unusual. The extremely limited number
23 of historical records of the occurrence of *S. hirsuta* in the region have been subsequently

1 attributed to misidentification of the hybrid (Stelfox 1947). An examination of herbarium
2 samples held at the National Botanic Gardens of Ireland, Glasnevin, found a single sheet
3 of *S. hirsuta* samples from the early 20th Century, labelled as a “garden escape” and
4 collected in the vicinity of Letterfrack, Co. Galway, which is on the periphery of the
5 current distribution of both *S. spathularis* and *S. x polita*. It is possible that this – or other
6 – garden plants might have contributed to the formation of populations of the hybrid.

7 Although *S. hirsuta* is generally far less common than *S. spathularis* in southwest
8 Ireland, where both species occur sympatrically, species distribution modelling under
9 future climatic scenarios indicated an increase in suitable habitat for the former relative to
10 its congener. Assuming the asymmetric introgression observed in the present study is
11 density-dependent, this would suggest that future introgression of *S. spathularis* alleles
12 into *S. hirsuta* may not be as pronounced as at present. However, two potential caveats
13 should be borne in mind. Firstly, it is well documented that such species distribution
14 models only take into account climatic factors and tend not to incorporate other biotic and
15 abiotic variables, such as species-species interactions (Pearson & Dawson 2003; Araújo
16 & Guisan 2006). Secondly, it is very possible that introgression of *S. spathularis* alleles
17 may lead to a shift in the ecological and/or climatic niche of *S. hirsuta*, thus
18 compromising these modelled potential future range expansions. Nevertheless, it is likely
19 that ongoing environmental change will lead to changes in ecosystem function, and this
20 could also be a factor at the level of the genome.

21 In conclusion, the levels of introgression observed in this study in both *S. spathularis*
22 and *S. hirsuta* would appear to be correlated with the relative abundance of the species.
23 This is reflected in the genetic composition of populations of the hybrid, *S. x polita*, and

1 its sympatric occurrence with one or both parental species. From a conservation
2 viewpoint, although significant levels of introgression of *S. spathularis* alleles were
3 detected in the majority of the *S. hirsuta* populations analysed, there were only a few
4 individuals in which *S. hirsuta* alleles were in a minority. This suggests that the *S.*
5 *hirsuta* morphology is being retained, despite varying degrees of introgression.
6 Nevertheless, ongoing introgression of *S. spathularis* alleles into *S. hirsuta* would appear
7 to represent a threat to the genetic integrity of the latter, particularly in areas where the
8 species exist sympatrically and where *S. spathularis* outnumbers *S. hirsuta*.

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2

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TABLE 1 Sites and numbers of samples collected. Multiple values indicate multiple patches sampled.

No	Location	Lat (N)	Long (W)	Number of samples		
				<i>S. spathularis</i>	<i>S. hirsuta</i>	<i>S. x polita</i>
1	Glengariff Forest Park	51.7539	9.5649	22 / 22 / 22	-	22
2	Glengariff Forest Park	51.7531	9.5652	8	-	8
3	Glengariff Forest Park	51.7533	9.5649	1	22	16
4	Glengariff Forest Park	51.7545	9.5694	7	11	11
5	Glengariff Forest Park	51.7562	9.5713	-	14	22
6	Glengariff Forest Park	51.7573	9.5724	22 / 4	15	15 / 8
7	Glengariff Forest Park	51.7551	9.5674	22	22	22
8	Glengariff Forest Park	51.7561	9.5713	1	8	8
9	Glengariff Forest Park	51.7566	9.5671	3	-	3
10	Glengariff Forest Park	51.7536	9.5613	5	19	16
11	Kenmare Tunnel	51.7560	9.5872	20	1	17
12	Beara Way	51.7072	9.6213	22	-	21
13	Beara Way Bridge	51.7049	9.6523	11	19	9 / 11
14	Dromagowlane	51.6886	9.6613	-	22	20
15	Lauraghbridge	51.7675	9.7705	9	8	4
16	Glenbeg Lough	51.7177	9.8737	22	6	12
17	Cleanderry Wood	51.7377	9.9247	-	22	-
18	Torc Mountain	51.9959	9.5083	20	-	15
19	Torc Waterfall	52.0067	9.5083	17	10	12
20	Meeting of the Waters	52.0048	9.5305	-	21	-
21	Old Torc Mountain Path	52.0045	9.5305	-	22	8
22	Gap of Dunloe	51.9918	9.6440	14 / 3	7	12
23	Ballaghbeama Gap	51.9407	9.8047	16	22	6
24	Camp	52.2152	9.9019	-	7	8
25	Owenmore River Bridge	52.2076	10.2084	1	10	8
26	Conor Pass	52.1818	10.2078	22	22	21
27	Connemara National Park	53.5514	9.9458	5	-	22
28	Clare Island	53.7974	10.0431	10	-	5
29	Mahon Falls	52.2324	7.5470	22	-	-
TOTAL				353	310	362

TABLE 2 *Saxifraga spathularis* / *S. hirsuta* AS-PCR primers

Locus	SNP	Flanking primers	SNP primers
3A09	G → A	AATATGTACTTTACCGTCCTC TGTGGGAAGTTCAGCATTG	CAAGTCAGGGAGGGGAG TGCACTACGTAAGTACCCT
3C09	4 bp indel	AATCTAAACAAACCCTAGAAAAC AAGTCCAAATATTTAAAAAATATATTTG	CATTCCAGATAAAATATGGCTAC AATTATGCCTAGACGAACTTG
3G12	C → T	TGTCTACTTTTTTCCCTATGC GTATCTATAAACACATATTTATGAAA	AATTTTTAATTTACACTAAAAACAGG TGTTAGTATATGAAATTGAGAGTTT
4C07	4 bp indel	CATGCCATATAACTTGATAATAC	GGTACGACTAAATCAACAATGG GGTATGGCTAAATCAACATTGA
4D05	C → T	GCACTCTCTCCCTGCACC	ATCTCAACGGTCAAAATTTATTC ATCTCAACGGTCAAACTTATTT
4F03	A → G	GATGTTCTGATTTTGAGAGAAG	TTTTTCCCTTTTTTCTGCCACA CTTCACCTTTTTTCTGCCACG
4H06	16 bp indel	CTAGATCGCCGGCGATC	CCTCTGTTATTCCAAATTGCG ACACACAGAACAACCTCTCTCTCTC

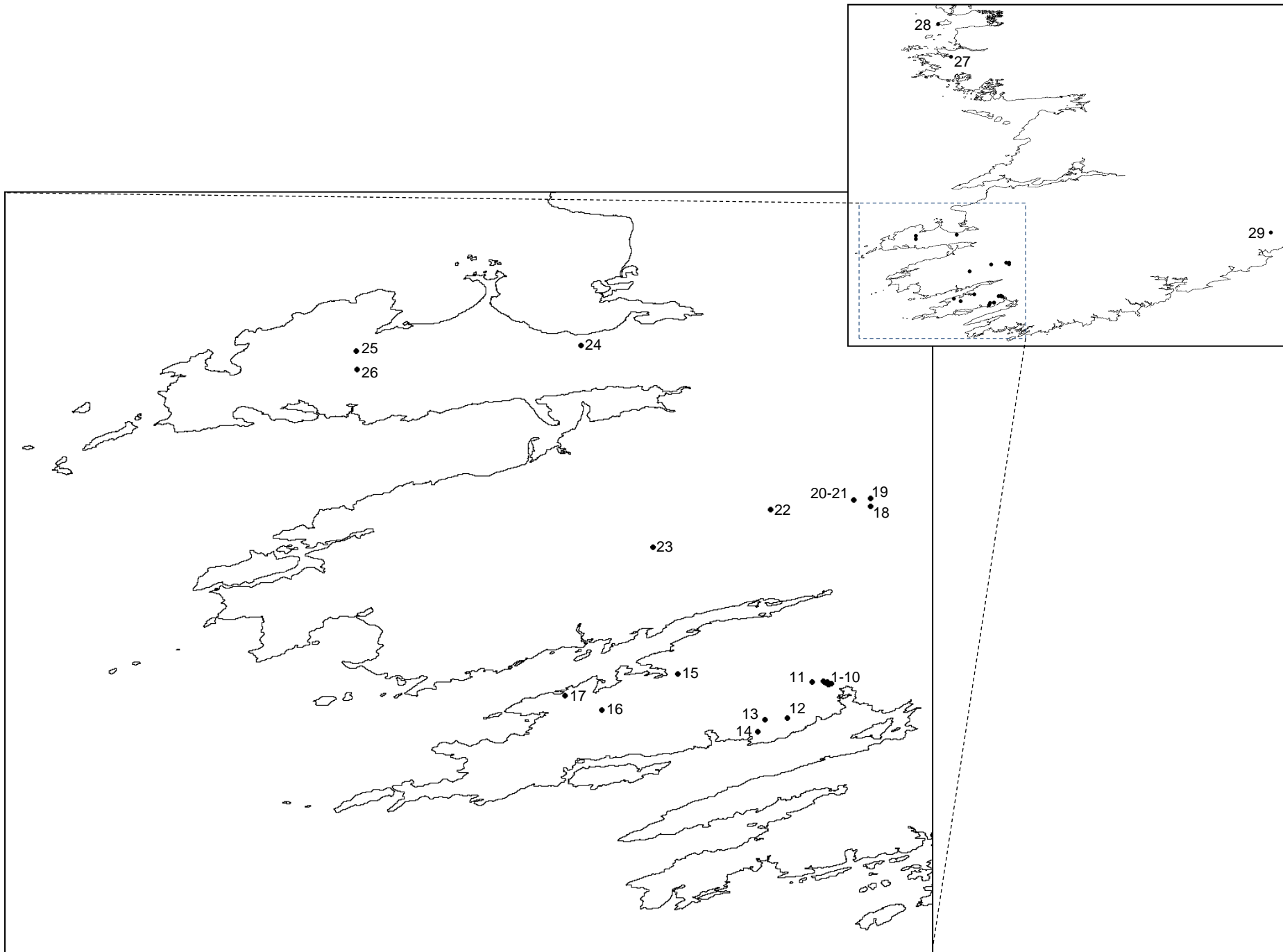
FIGURE LEGENDS

FIG. 1 Maps showing locations of populations sampled in this study. Numbers refer to populations listed in Table 1.

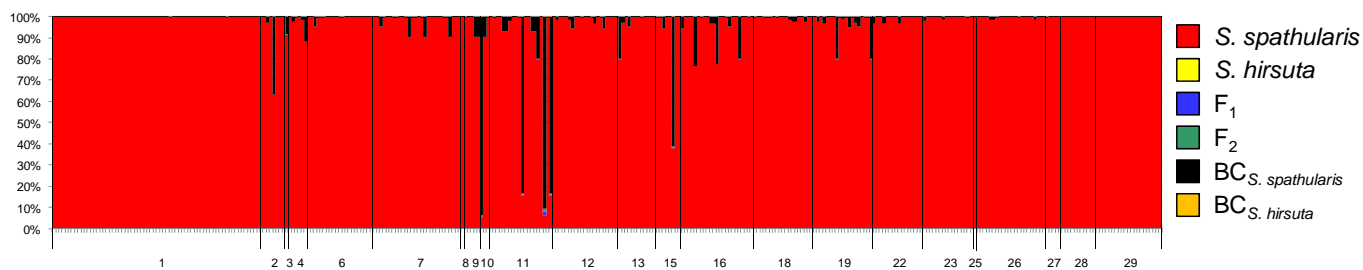
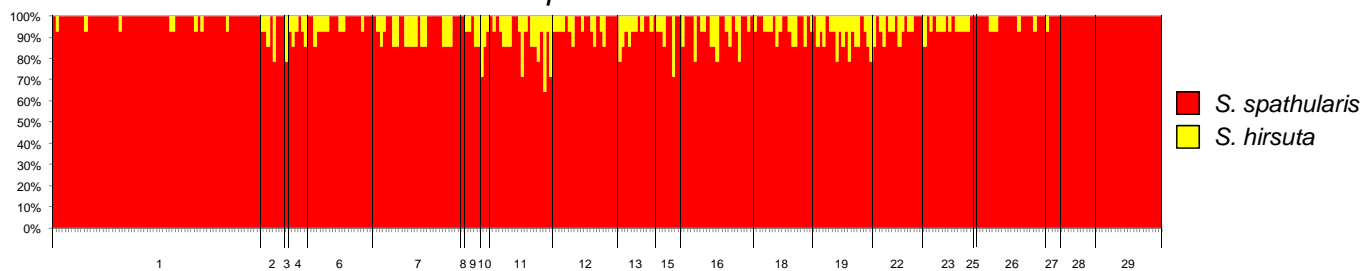
FIG. 2. Allele frequencies (above) and results of the NEWHYBRIDS analysis (below) for each of the three taxa studied. In all diagrams, each column represents a single individual. The length of each coloured segment in the NEWHYBRIDS is proportional to the Bayesian posterior probability of assignment to the corresponding genotypic class. Numbers refer to populations in Figure 1 and Table 1.

FIG. 3. Species distribution models for *S. spathularis* (a-c) and *S. hirsuta* (d-f). (a,b) 2000; (c,d) 2050; (e,f) 2080.

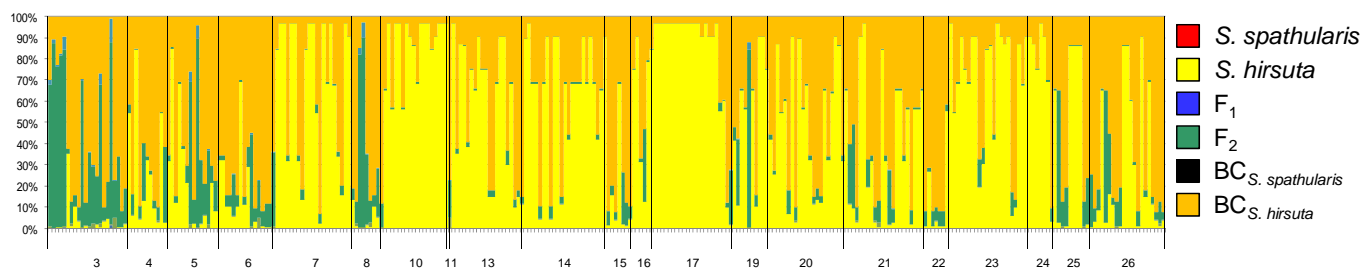
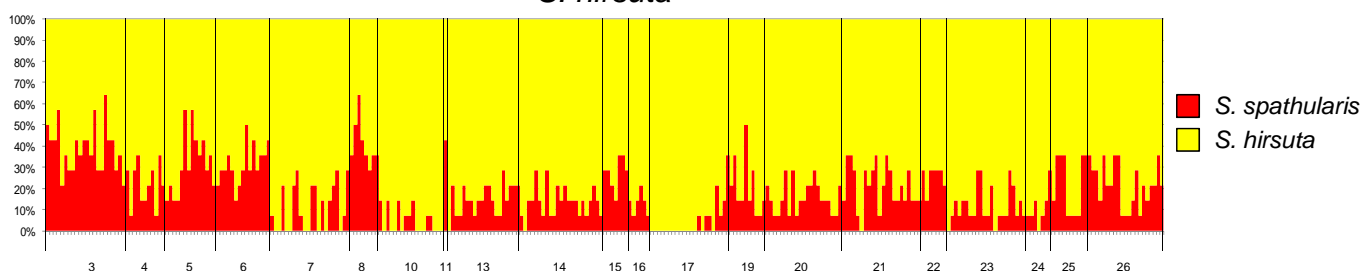
FIG. 4. Number of climatically suitable cells predicted by the species distribution models for *S. spathularis* and *S. hirsuta* in southwestern Ireland for each of three time periods.



S. spathularis



S. hirsuta



S. x polita

